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Short communication

Acute and persistent infection by a transfected Mo7 strain of *Babesia bovis*

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ABSTRACT

A Mo7-derived *Babesia bovis* line stably transfected with the *gfp-bsd* gene was inoculated into two four to five months old calves, while two additional calves were inoculated with Mo7 parasites. Similar mild clinical signs were detected in all calves. *B. bovis rap-1* was identified in the bloodstream by PCR four days post inoculation (dpi), and consistently over ten months thereafter. Transfusion of blood from experimentally infected calves into four naïve splenectomized calves at 212 dpi resulted in acute disease in recipients, confirming persistent infection in the four donor animals. The proportion of GFP expressing parasites recovered from a splenectomized recipient calf is undistinguishable from transfected parasites that were maintained in long term culture under blasticidin selection. Furthermore, the sequences of transfected genes in recovered parasites remained unaltered. Together, the data demonstrates that exogenous *B. bovis* transgenes can be expressed and remain stable throughout acute and persistent infection in calves.

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Bovine babesiosis is a tick-borne intraerythrocytic protozoal disease of global impact [1]. *Babesia bovis* transmitted primarily by *Rhipicephalus* (*Boophilus*) *microplus* ticks causes acute fulminant disease with high mortality. Animals that survive acute infection remain persistently infected [1,2]. *B. bovis* parasites can be attenuated by serial passage in splenectomized calves, and attenuated parasites are currently the only effective immunization method available to prevent clinical disease caused by virulent *B. bovis* strains [2].

An important limitation of vaccination with live parasites is the lack of a reliable, direct and simple method to discriminate between vaccinated and naturally infected animals in endemic areas. One approach toward addressing this limitation is development of marker vaccines using recombinant approaches. Because attenuated parasites also establish persistent infection, expression of exogenous genes by stably transfected, attenuated *B. bovis* provides an opportunity to explore the possibility of combining antigens from multiple infectious agents and parasites into a single live vaccine. The ability to persistently infect the host makes this approach particularly promising when immune protection requires frequent boosting, for example, with other hemoparasites and ticks [3,4]. However, to serve as efficient live vectors for vaccination, recombinant attenuated *B. bovis* vaccines would be required to establish mild acute infection leading to persistence, express exogenous antigens throughout infection, and remain genetically stable. Several laboratories, including our own, have been able to stably transfect *B. bovis* [5–7], removing a technical barrier for creating recombinant marker and multivalent attenuated vaccines. However, previous work has not demonstrated the requisite *in vivo* features of such vaccines in cattle, the natural host of *B. bovis*. The study described herein addresses these requisite features using a stably transfected, attenuated strain of *B. bovis*.

A *B. bovis* Mo7 [8,9] transfected strain termed Tf-137-4 was produced for the purpose of this study using the transfection plasmid construct *pgfp-bsd-ef* as described previously [6]. Mo7, a biologically cloned strain [8], and Tf-137-4 *B. bovis* parasites were maintained in *in vitro* cultures [9,10]. Following transfection, a drug-resistant transfected line emerged eight days after the onset of selection with blasticidin. The parasite line Tf-137-4 was examined repeatedly over 6 months of *in vitro* culture as previously described [6] using fluorescence microscopy, reverse transcriptase PCR and Western blot to confirm continuous expression of the *gfp-bsd* gene. Fluorescence analysis of the Tf-137-4 line showed distinctive intracellular fluorescence, with a pattern similar to previously described transfected parasites [6] (refer to Fig. 3A, panel 2). Integration of the *gfp-bsd* insert into the *B. bovis ef-1a* locus was demonstrated using Southern blots, direct PCR and sequence

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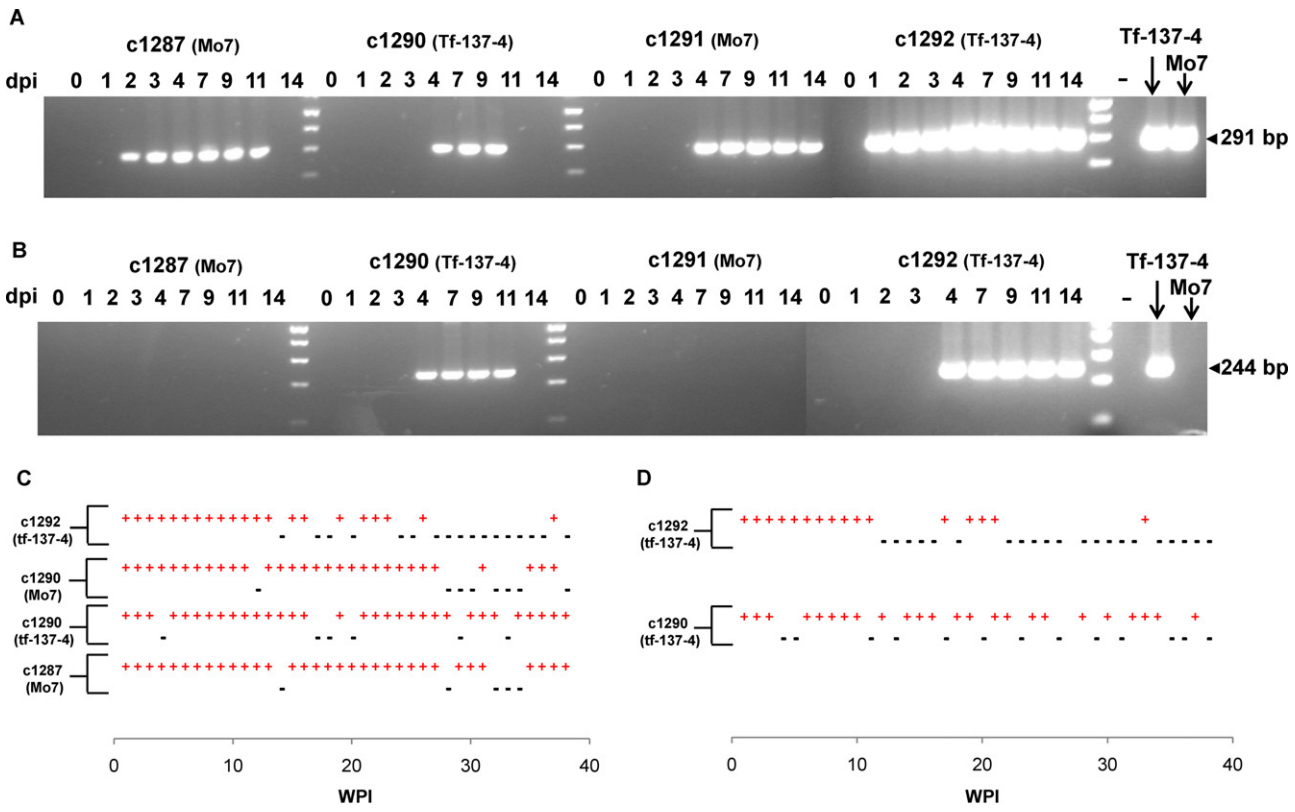

Fig. 1. Detection of *B. bovis rap-1* DNA and the *gfp-bsd* genes in experimentally infected calves. Panel A: nested PCR amplification of a 291 bp fragment of the *rap-1* gene. Panel B: nested PCR amplification of a 244 bp fragment of the *gfp-bsd* gene. PCR reactions were performed using DNA extracted from the blood of all four experimentally infected animals at days 0–4, 7, 9, 11, and 14 days post-inoculation (dpi) with either the *B. bovis* Mo7 strain (c1287 and c1291) or the *B. bovis* transfected Tf-137-4 parasite line (c1290 and c1292). Negative control PCR amplifications (−) were performed using identical sets of primers as the positive control reactions, but without the addition of DNA. Positive control amplifications were performed on DNA extracted from cultured Mo7 and Tf-137-4 parasites. Panel C: nested PCR amplification of a 291 bp fragment of the *rap-1* gene on total DNA extracted from the blood of experimentally infected animals performed at different weeks post-inoculation (WPI) (*x*-axis). Positive nPCR reactions are marked as a red (+) sign and negative nPCR reactions as a black (−) sign. Panel D: nested PCR amplification of a 244 bp fragment of the *gfp-bsd* gene on total DNA extracted from the blood of experimentally infected animals performed at different weeks post-inoculation (WPI) (*x*-axis). Positive nPCR reactions are marked as a red (+) sign and negative nPCR reactions as a black (−) sign. Individual animals are designated as follows: c1287 (Mo7), c1290 (Tf-4-137), c1291 (Mo7), and c1292 (Tf-4-137).

analysis as described [6]. The Tf-137-4 line was cultured for 6 months to confirm stable integration.

Cultured Tf-137-4 parasites were used for experimental infection of two spleen-intact Holstein calves (c1290 and c1292) aged four to five months, while 2 additional calves (c1287 and c1291) were inoculated with cultured parasites of the parental wild type Mo7. The protocol of infection used in this study and all animal handling, was approved by the Institutional Animal Care and Use Committee, IACUC, Washington State University (#03735-008 approved on 12/9/2009). Prior to inoculation, all calves tested negative for the presence of pre-existing infections with *B. bovis* using nested PCR (nPCR) and cELISA [11]. All calves received 5×10^3 infected erythrocytes intravenously [12] and were monitored for signs of acute babesiosis, including parasitemia, fever, and packed cell volume (PCV) on a daily basis for the first thirty days post-inoculation (dpi) and on a biweekly basis thereafter. Maximum decreases in the PCV ranged from 23% to 33% among the four inoculated animals and occurred at seven or eight days after inoculation (Supplementary Table S1). Statistical analysis (*T*-test) showed no significant difference in the maximum decrease of PCV between the animals infected with the wild type parasite line Mo7 as compared to those infected with the transfected parasite line Tf-137-4. In addition, changes in rectal temperatures, which increased in the four animals at seven days after inoculation (ranging from 1.5 to 2.17 °C), were not significantly different among the two experimentally infected groups (Supplementary Table S1). Intraerythrocytic parasites were first evident seven days after the date of inoculation and the percentage of parasitized erythrocytes in the bloodstream (PPE) was less than 0.01% in all animals. Parasites were undetectable by direct microscopy by 12 dpi. Neither hemoglobinemia nor hemoglobinuria was detected in the infected animals during the acute stage of the disease, and severe babesiosis, characterized by recumbency and neurological signs, was not observed. We concluded that both parental and transfected Mo7 strain *B. bovis* caused mild acute clinical babesiosis in this experiment with no differences attributable to an infecting strain and which were consistent with previous studies of cattle infected with the Mo7 strain [9,13], All four experimentally infected calves recovered from the mild acute stage of the disease and returned to normal clinical parameters (PCV, fever) by thirty days after the onset of experimental infection (data not shown). To determine the persistence of the infection, we examined whether *B. bovis* DNA could be detected in the blood of the four experimentally infected calves. Nested PCR (nPCR), on total DNA extracted from peripheral blood was performed using the following *rap-1* primers as previously described [11]: Rap-1-Ext-Forward *BoF* (5′-CAC GAG GAA GGA ACT ACC GAT GTT GA-3′), Rap-1-Ext-Reverse *BoN* (5′-CCA AGG AGC TTC AAC GTA CGA GGT CA-3′), and Rap-1-Nested-Forward *BoFN* (5′-TCA ACA AGG TAC TCT ATA TGG CTA CC-3′), Rap-1-Nested-Reverse *BoRN* (5′-CTA CCG AGC AGA ACC TTC TTC ACC AT-3′). A 291 bp *rap-1* amplicon could be detected by day four post-inoculation in all animals (Fig. 1A). In addition, nPCR amplifications was performed using *gfp-bsd* specific primers *Gfp-Forward-EcoRV* (5′-CGC GTA GAT ATC ATG GCC TCC AAA GGA GAA GAA C-3′), and *Bsd-Reverse-EcoRV* (5′-GTA CCG GAT

ATC GCC CTC CCA CAC ATA ACC AGA GG-3′), and the nested primer set *GFP-N-F2*: (5′-TGG AAA CAT TCT CGG ACA CA-3′), and *GFP-N-R2* (5′-CTT TTC GTT GGG ATC TTT CG-3′). Strong 244 bp amplicon signals were consistently detected at 4 dpi only in the Tf-137-4-recipient steers c1290 and c1292 (Fig. 1B). All amplicons obtained in the nPCR amplifications were sequenced and in all cases the sequences were identical to the *rap-1* or the *gfp-bsd* sequences amplified from control Mo7 and *gfp-bsd* plasmid DNA. Nested PCR to detect the *rap-1* and *gfp-bsd* genes was also performed on total DNA obtained from the blood of all infected calves throughout the course of the infection on a weekly basis (Fig. 1C and D). Parasite DNA was detected in all animals (Fig. 1C). Amplicons of the *gfp-bsd* gene (Fig. 1D) were obtained up to ten months after the start of the infection for animal c1290 and for approximately 8 months for animal c1292. The periodic failure to detect parasites by nPCR in some weeks is typical during the cyclic parasitemia that occurs in persistently infected animals [14]. Sequences of the *gfp-bsd* 244 bp nPCR amplicons were identical to the sequences in the Tf-137-4 line used for experimental inoculation (data not shown).

We performed RT-PCR analyses for *rap-1*, *gfp-bsd* gene sequences on total RNA extracted from peripheral blood using nested RT-PCR. Transcription of the *B. bovis rap-1* gene was detected by nested PCR using primers *BoF*, *BoRN*, *BoFN*, and *BoN* in multiple samples as expected (data not shown). Transcription of the *gfp-bsd* gene was detected by nested RT-PCR (primers *Gfp-Forward-EcoRV*, *Bsd-Reverse-EcoRV*, *GFP-N-F2* and *GFP-N-R2*) on blood obtained from calf c1292, but not calf c1290 (both infected with the Tf-137-4 transfected strain) on 45 and 52 dpi (Supplementary Fig. S1). The sequence of the 244 bp *gfp-bsd* cDNA amplicon was identical to the transfected *gfp* gene sequence in the Tf-137-4 transfected strain (data not shown). These data demonstrate transcription of the transfected gene during infection of calves.

The very low level of parasitemia in these persistently infected cattle presented technical challenges in detecting transcripts and analyzing genomic changes in greater detail. Thus, to confirm the establishment of persistent infection in all experimentally infected animals and to expand parasite populations for additional analysis, 50 ml of whole blood from each infected animal was transfused at 212 dpi by syringe passage to different splenectomized recipient calves: c31008 and c30870 were inoculated with blood from Mo7-recipients c1287 and c1291, whereas the recipient calves c31026 and c30872 were inoculated with blood from Tf-137-4 recipients c1290 and c1292, respectively. Two of the splenectomized recipient calves (c31008 and c31026) were euthanized twelve days post-transfusion due to the development of severe acute babesiosis, including a 45% decrease in PCV (to 14%), fever above 38.8 °C for two consecutive days, and a PPE of 0.5%. The other two splenectomized recipient calves (c30870 and c30872) developed only mild clinical babesiosis and remained under study for an additional 3 months. Parasites were identified in all splenectomized recipient calves, confirming that all spleen-intact donor calves were persistently infected with viable *B. bovis* parasites.

RT-PCR analyses for *rap-1*, *gfp-bsd*, and *ef-1α* gene sequences were performed on total RNA extracted from peripheral blood of the two splenectomized recipient calves post-euthanasia. Fresh and 10% buffered neutral formalin-fixed brain tissue was collected at post mortem examination. Histological examination of fixed tissues revealed a high percentage of parasitized cells in cerebral capillaries with no differences between the parental and transfected strains (data not shown). Total DNA extracted from blood and frozen unfixed cerebral tissue, together with cDNA derived from total RNA extracted from the same tissues, were amplified using *rap-1* primers *BoF* and *BoN* and the expected 356 bp amplicon was detected (Fig. 2A and B). When *gfp-bsd* primers *gfp-Forward-EcoRV* and *Bsd-Reverse-EcoRV* were used for cDNA and total DNA PCR amplifications, a 1128 bp amplicon identical in sequence to the *gfp-bsd* gene was obtained from c31026 (Tf-137-4-infected blood recipient), but not from c31008 (Mo7 infected blood recipient) (Fig. 2A and B). In addition, when control RT-PCR amplification of the full size *orf* of the *ef-1α* gene (1343 bp) was performed using primers *EF-Bo-F* (5′-ATG CCG AAG GAG AAG ACT CAC-3′) and *EF-Bo-Rev* (5′-CTT CTT AGC AGC CTT TTG GGC-3′), the expected 1343 bp band was obtained for both animals (Fig. 2A and B). Control PCR amplifications performed on total DNA extracted from blood and cerebrum, together with plasmid and total *B. bovis* genomic DNAs are shown in Supplementary Fig. S2. Sequence analysis of all RT-PCR amplicons confirmed full identity with the *rap-1*, *gfp-bsd* and *ef-1α* gene sequences, demonstrating stability of the transfected genes. Because the expected PCR and RT-PCR bands were produced using total DNA and RNA extracted from cerebrum and peripheral blood, we confirmed the presence of transfected parasites in c31026 that retained and transcribe the *gfp-bsd* gene *in vivo* in the absence of the selective pressure of blasticidin.

The transfected *B. bovis* strain Tf-137-4 was culture-recovered from the blood of splenectomized recipient c31026 and termed the CR-Tf-137-4 parasite line. Blasticidin resistant fluorescent parasites were detected seven days after the establishment of the *in vitro* cultures (Fig. 3A) confirming that the recovered parasite line retains the ability to express the *gfp-bsd* gene after 212 days of replication *in vivo* in the absence of blasticidin selection pressure. Subjective visual assessment of Mo7 and CR-Tf-137-4 parasites labeled with an anti-GFP fluorescent-conjugated antibody and DAPI nuclear stain suggested no anti-GFP labeling of *B. bovis* Mo7 parasites and complete labeling of the CR-Tf-137-4 parasites (Supplementary Fig. S3A and B). However, to more objectively determine the proportion of the CR-Tf-137-4 parasites expressing GFP-BSD, we evaluated the extent of anti-GFP labeling using a densitometric approach (Supplementary Fig. S3C, Supplementary Methods, and Supplementary Table S2).

Briefly, multiple 20× fields of view of each slide preparation of CR-Tf-137-4 parasites (cultured either in the presence or the absence of inhibitory doses of blasticidin [Supplementary Fig. S3A]), Mo7 parasites, or Tf-1-2-124 parasites (a *B. bovis* line not passaged in cattle but kept continuously in culture in the presence of inhibitory doses of blasticidin for more than three years [Suarez and McElwain [6]]) were digitally acquired using the same exposure settings and in one imaging session. Threshold generated regions of interest were created for each organism stained by DAPI and the integrated density of DAPI and anti-GFP fluorescence channels measured. As expected from visual assessment of the slides, the data formed two well-separated elliptical density groups of high (Tf-1-2-124 and CR-Tf-137-4, pre-and post-selection) *versus* low (Mo7) anti-GFP labeling (Supplementary Fig. S3C). As objectively determined using a two-step cluster analysis (Supplementary Table S2), only 1.33% (95% exact confidence limits: 0.69–2.31%) of the positive control parasite line Tf-1-2-124, were categorized as low anti-GFP labeling, the same cluster group containing 100% of the negative control parasite line, Mo7. Similarly, low anti-GFP labeling was detected in only 0.52% (0.11–1.51%; pre-selection with blasticidin) and 1.26% (0.55–2.47%; post-selection) of the cattle passaged line, CR-Tf-137-4. As determined by logistic regression, the odds ratios for anti-GFP labeling cluster group assignment were not significant ($P > 0.1$) for comparisons of CR-Tf-137-4 (pre- and post-blasticidin) and Tf-1-2-124 parasite lines, but were significant ($P < 0.0001$) between any of these GFP-transfected parasite lines and the non-transfected Mo7 strain. Taken together, these data demonstrates that the CR-Tf-137-4 parasites recovered from the splenectomized recipient c31026 retain their ability to express the exogenous GFP-BSD protein in a pattern that is undistinguishable from Tf-1-2-124 parasites maintained in *in vitro* culture, regardless of growing in the presence or absence of blasticidin. Thus we concluded that two *in vivo* passages of the transfected Tf-137-4 parasite

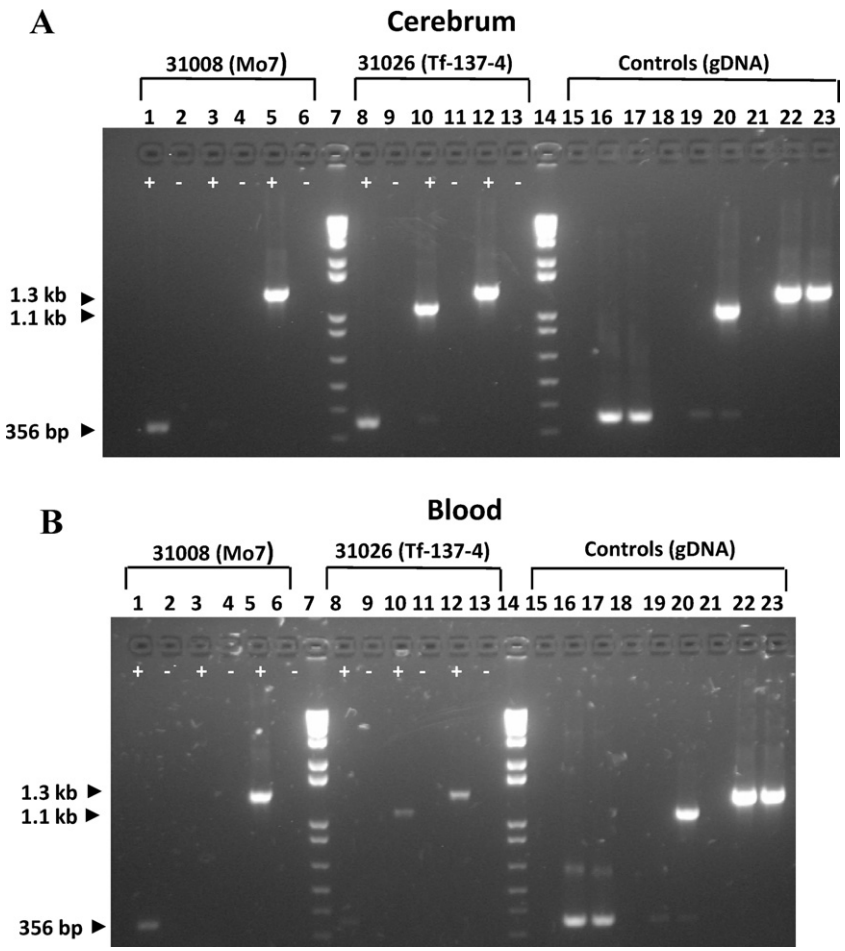

Fig. 2. Analysis of acutely infected splenectomized recipient steers c31008 and c31026 inoculated with blood from the Mo7 and the Tf-137-4 *B. bovis* persistently infected animals, respectively. Panel A: detection of *rap-1* and *gfp-bsd* transcripts in total RNA extracted from cerebrum by RT-PCR. Panel B: detection of *rap-1* and *gfp-bsd* transcripts in total RNA extracted from peripheral blood by RT-PCR. PCR amplifications after reverse transcription and control amplifications without reverse transcriptase are marked as (+) or (−) respectively. Lanes 1, 2, 8, and 9 represent amplifications with *rap-1* primers; lanes 3, 4, 10, 11,18, 19 and 20 represent amplifications with *gfp-bsd* primers; lanes 5, 6, 12, 13, 21, 22, and 23 represent amplifications with *ef-1α* primers. Control PCR amplifications performed on genomic DNA on lanes 15, 18, and 21 were done on total DNA extracted from uninfected steers, on lanes 16, 19, and 22, on DNA from cultured Mo7 parasites, and lanes 17, 20 and 23, on total DNA from *in vitro* cultured transfected strain Tf-137-4.

line resulted in no significant change in the retention or expression levels of GFP-BSD.

To determine whether sequences of the transfection construct remained stable during the course of the *in vivo* infection, we generated and sequenced three overlapping DNA amplicons representing sequences in the site of insertion of the transfected genes and the entirety of the transfected sequences encoded in plasmid *pgfp-bsd-ef* which was used to transfect the parasites [6] (see Fig. 3B), from genomic DNA extracted from CR-Tf-137-4 *B. bovis* parasites. Amplicon 1 was generated using primers *ef-rev5* (5′-CAT ATC AAG CTT CTT TAA CGG GAT GAC ATA TAT G-3′) and *ef-rev6* (5′-GAC CAT AAG CTT AGT AAA CGA TAG AAC AGA CTA AG-3′) [6]. Forward primer *ef-rev-6* is targeted to sequence unique to the intergenic region of the *ef-1α* locus in the Mo7 wild type parasites, but not present in the transfection plasmid, and the reverse primer *ef-rev-5* represents sequences in the *ef-1α* promoter of the *gfp-bsd* gene of the transfection vector *pbsd-gfp-ef*. Amplicon 2 was generated with primers *EF-orf-F1* (5′-CTG ACG CTC GAG ATG CCG AAG GAG AAG ACT CAC-3′), and *Trancon-rev* (5′-TCC TTT AGT GAG GTT CAC G-3′) that target a ∼2.8 kbp fragment present only in the transfection plasmid *pgfp-bsd-ef* (Fig. 3B) [6]. Amplicon 3 was generated with primer *pst-IgF* (5′-GCT ACT CTG CAG GAT GAG ATG CGT TTA TAA T-3′) and primer *ef-loc-rev* (5′-TAC GCT TTC CCT GTT TGT AAC-3′). Forward primer *pst-IgF* represents sequences in the *rap-1* 3′region of the transfection vector *pbsd-gfp-ef*, and primer *ef-loc-rev* is targeted to sequence unique to the *glutamyl tRNA synthase* gene which is located immediately 3′ to the *ef-1α-B* gene [15] in wild type parasites, but not present in the transfection plasmid. The sequences in the three overlapping amplicons were assembled into a single 5996 bp contig termed Tf-137-4-insertion (GenBank Accession number: JQ946523), that is represented in Fig. 3B. Sequence comparisons demonstrated that sequences in amplicons 1, 2 and 3 generated from genomic DNA of CR-Tf-137-4 parasites are identical to the equivalent sequences in the transfection plasmid *pgfp-bsd-ef*. Furthermore, the sequence of amplicon 1 was identical to the previously identified insertion sequence in the transfected *B. bovis* line Tf-1-2-124 (GenBank Accession number: ACG69233.1) [6] and the 3′ portion of the insertion present in the amplicon 3 contains sequences of the *glutamyl tRNA synthase* gene which are not present in the transfection plasmid *pgfp-bsd-ef*. Thus, analysis of the sequences inserted in the genome of CR-Tf-137-4 parasites confirms the location, identity, and stability of the transfected sequences integrated into the *ef-1α* locus in transfected parasites.

Taken together the results demonstrate that transfection of the Mo7 strain, as performed in this study, does not appear to cause significant fitness costs to the parasite, and results in a stable construct with the characteristics required of a recombinant attenuated *B.*

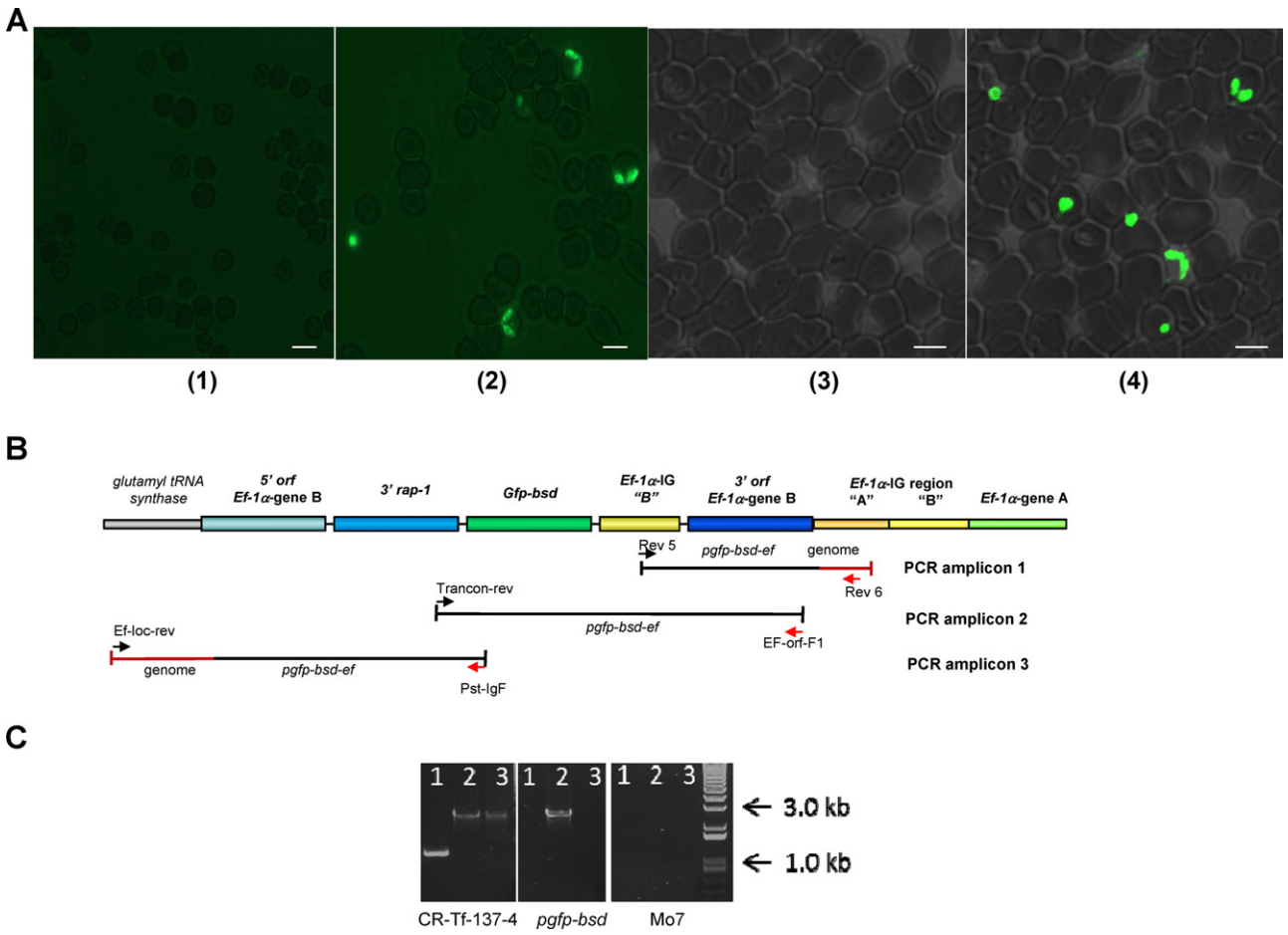

Fig. 3. Panel A: detection of GFP-BSD in *B. bovis* by (1 and 2) fluorescence microscopy, and (3 and 4) microscopic analysis by differential interference contrast (DIC) microscopy. Microscopic analysis was performed on cultured *B. bovis* Mo7 infected cells (1 and 3), and transfected parasite line Tf-137-4 (2) and CR-Tf-137-4 (4). The GFP-BSD protein is detectable only in transfected parasites. White bars at the right bottom of each panel represent 5 μm. Panel B: schematic representation of the insertion of sequences in plasmid *pgfp-bsd-ef* integrated into the *ef-1α* locus of transfected CR-Tf-137-4 parasites recovered from c31026 (GenBank accession number: JQ946523), and of amplicons 1, 2, and 3. The relative location of sequence identical to primers *rev5* and *rev6* used for generating amplicon 1, *Ef-orf-f1* and *Trancon-rev* used for generation of amplicon 2, and primers *Ef-loc-rev* and *Pst-IgF* used for amplicon 3 are represented with arrows. Panel C: PCR amplification of transfected genes using DNA extracted from CR-Tf-137-4 parasites, plasmid *pgfp-bsd-ef*, and the *B. bovis* Mo7 strain. Lane 1, amplifications using primers *rev5* and *rev6* (amplicon 1, size: ~1.4 kb); lane 2 amplifications using primers *ef-f* and *Trancon-rev* (amplicon 2, size: ~2.8 kb); lane 3 primers *Ef-loc-rev* and *Pst-IgF* (amplicon 3, size: ~2.8 kb). The arrow on the right marks the positions of the 1.0 and 3.0 kbp size markers in the gels.

bovis vaccine. Though cattle inoculated with Mo7 parasites are protected from clinical disease after challenge with virulent strains [13], it remains to be determined if a recombinant vaccine based on the Mo7 attenuated strain is also able to confer protection against challenge with virulent strains and whether the transfected gene can be used as a vaccine marker.

This is the first demonstration of the *in vivo* characteristics of stably transfected *B. bovis*. Experiments to optimize transgene expression, particularly during persistent infection, and to determine the tick transmissibility, immunogenicity, and protective ability of recombinant vaccine strains of *B. bovis* expressing foreign antigens are currently underway.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.molbiopara.2012.05.003.

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